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Regulatory Analysis of Vimentin Expression in Metastatic Versus Nonmetastatic Breast Cancer Cell Lines

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REGULATORY ANALYSIS OF VIMENTIN EXPRESSION IN METASTATIC VERSUS NONMETASTATIC BREAST CANCER CELL LINES

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

By

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List of Abbreviations

bp	base pair
$CaPO_4$	calcium phosphate
CAT	chloramphenicol acetyltransferase
DMEM	Dulbecco's Modified Eagle Medium
cpm	counts per minute
HBSS	Hanks' Balanced Salt Solution
IF	intermediate filament
IFP	intermediate filament protein
kb	kilobase
kDa	kilodalton
M	molar
MDA	MDA-MB-231
MEM	Minimum Essential Medium
p	plasmid
pol II	eukaryotic RNA polymerase II
pg	picogram
pm	picomole
µg	microgram
µl	microliter

ABSTRACT

REGULATORY ANALYSIS OF VIMENTIN EXPRESSION IN METASTATIC VERSUS NON-METASTATIC BREAST CANCER CELL LINES

By Danielle Nichole Bird, B.A.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 1994.

Major Director: Dr. Zendra E. Zehner, Ph.D.
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The intermediate filament gene family, composed of six classes, shows both tissue and development-specific expression. Vimentin is a unique member of the intermediate filament protein family: although it is expressed in cells of mesenchymal origin, vimentin may also be expressed with other intermediate filament proteins during early stages of development. In some cases, as differentiation continues,

vimentin is normally down-regulated whereas other intermediate filament proteins, like desmin, glial fibrillary acidic protein, or neurofilaments are turned on in muscle, glial cells, or neurons, respectively. In some metastatic cancers, including breast and prostate cancers, vimentin is aberrantly expressed, despite the embryonic origin of the metastatic cell. From a Northern analysis (Thompson et al., 1992; Stover et al., 1994), it was determined that vimentin mRNA is highly abundant in the metastatic breast carcinoma cell line, MDA-MB-231, while in the nonmetastatic breast carcinoma cell line, MCF-7, no vimentin mRNA is present.

A central question to metastasis is, how is the vimentin gene expressed in the metastatic but not the nonmetastatic cancer cell? Several *cis*-acting elements localized to the 5'-end of the chicken vimentin gene and *trans*-acting factors important in the regulation of vimentin gene expression have been identified. By sequence homology, comparable elements can be found in the human vimentin gene. Most notable of these is a unique silencer element and an overriding element, referred to as an antisilencer. These

elements bind a 90 kDa and 120 kDa protein, respectively, in chicken, mouse, and human nuclear extracts. Previously, the silencer factor was found to be missing in nuclear extracts from a metastatic breast cancer cell line MDA-MB-231 but abundant in the nonmetastatic counterpart MCF-7 (Stover et al., 1994). The antisilencer exhibits the opposite pattern. Here, various combinations of these elements have been fused to the bacterial chloramphenicol acetyltransferase reporter gene, CAT. Transcriptional activity was then compared in the different breast cancer cell lines in order to try and understand how vimentin is expressed in the metastatic but not the nonmetastatic cancer cell.

INTRODUCTION

The goal of this thesis is to examine the expression patterns of various regulatory regions from the chicken vimentin gene in metastatic versus nonmetastatic human breast cancers. Specifically, functional activity will be analyzed in MDA-MB-231 cells, which express vimentin, and in MCF-7 cells, which do not express vimentin. By using various combinations of the 5'-end regulatory regions, it may be possible to conclude which region(s) is responsible for controlling the aberrant expression of vimentin in the MDA cell line. To fully understand this project, several issues need to be addressed. These include the differences in the two cell lines chosen for this study and the regulatory regions in the 5'-end of the vimentin gene that have been thus far characterized. Also, an overview of the expression pattern of the vimentin protein, transcriptional processes, and the intermediate filament protein (IFP) family will

complete the discussion. Since this study is concerned with the regulation of the IFP vimentin, it is appropriate to begin with a description of intermediate filaments (IF).

Intermediate Filament Proteins

The cytoskeleton of cells is composed of three types of filaments: microtubules (25 nm in diameter), intermediate filaments (11 nm in diameter), and microfilaments (6 nm in diameter). Of these, the intermediate filament proteins (IFPs) are the major component of this network and serve several functions. They are believed to anchor the cell's organelles, including the nucleus, as well as to maintain compartmentalization and possibly communication within the cell (Steinert and Roop, 1988; Steinert and Liem 1990).

Vimentin belongs to one of six different IF gene classes. These classes include: acidic keratins (Type I), neutral-basic keratins (Type II), vimentin, desmin and glial fibrillary acidic proteins (Type III), neurofilaments (Type IV), lamins (Type V), and nestins (Type VI) (Steinert and Roop, 1988; Lendahl et al., 1990).

In general, the IFs have been found to be tissue-

specific and developmentally regulated. Vimentin is unique in that it deviates from the normal bounds of the IF expression pattern. Although it is usually found in cells of mesenchymal origin, vimentin may also be coexpressed with any of the other IFPs early in cellular differentiation. For example, in developing astrocytes, vimentin may be coexpressed with glial fibrillary acidic proteins (Schnitzer, J. et al. 1981; Tapscott, S.J., et al., 1981; Yen, S.-H., and K.L. Fields, 1981). During myogenesis, when the myoblast differentiates to the myotube, vimentin is down-regulated while the expression of desmin is up-regulated (Sax et al., 1989; Bennett et al., 1979; Capetanaki et al., 1984; Holtzer et al., 1982). As in the case of the aforementioned breast cancer, various tumors of non-mesenchymal origin may coexpress vimentin (McGuire et al., 1989). The regulation of vimentin must therefore be complex and is under current investigation.

Intermediate Filament Protein Structure

IFPs have a common general secondary structure due to the highly conserved central rod domain. The α-helical rod

is flanked by the N-terminus, commonly referred to as the head region, and by the C-terminus, commonly referred to as the tail region.

The head region accounts for most of the deviations in amino acid sequence between IFPs and between species. Although not quite as diverse, the tail region also exhibits little similarity between IFPs and between species. The central rod, the region with the most conservation, has a common quasi-repeating heptad of amino acids, delineated a-b-c-d-e-f-g. Positions a and d are usually apolar residues while b,c,e and g are usually polar (Zehner et al., 1986; Ngai et al., 1990). This primary sequence allows the IFP subunits to wrap around each other, forming a basis for the coiled-coil structure characteristic of IFPs.

Intermediate Filament Protein Assembly

The first level of assembly is the formation of dimers. Dimers of the IFPs are formed by the coiled-coil interactions between the α-helical rod portions. The hydrophobic short-chain amino acids at positions a and d of the heptad repeat allow the chains to pack close together in

forming the dimer. These interactions are coordinated by the flanking head and tail regions which are thought to wrap around and stabilize dimer formation. Next, the dimers are paired in an anti-parallel fashion to form a tetramer, called a protofilament. Due to the anti-parallel pairing, the protofilaments are nonpolarized (Ngai et al., 1990).

At the next step of assembly, protofilaments are thought to form an axially staggered array called a protofibril. Each fibril is equivalent to two protofilaments. The final 11 nm-diameter filament is formed by the association of four protofilaments.

In instances where vimentin is co-expressed with other IFPs, it is not uncommon to find random heteropolymers. Although it has been demonstrated that IFPs will spontaneously polymerize *in vitro*, a simple self-assembly mechanism is highly unlikely since de novo assembly has been disproved (Ngai et al., 1990). Based on the observation that newly synthesized vimentin is incorporated into preexisting filaments, Ngai et al. has proposed a directed self-assembly scheme. According to this theory, assembly-competent regions within filaments are established or maintained to allow free

subunits to assemble and elongate the filaments (Ngai et al., 1990). This theory is currently supported by the observation that vimentin subunits will insert at various discreet loci in the cytoplasm (Coleman and Lazarides, 1992).

Presently, there are over thirty different IFPs, and new members are still being isolated. For the most part, each IFP arises from its own unique gene: there is only one known example of differential splicing giving rise to more than one type of IF. Therefore, differential gene expression patterns must have evolved coincident with IFP divergence. Based on this, the regulation of eukaryotic gene transcription is important to determining which IFP is found in a particular cell type.

Eukaryotic Transcription

In general, transcription initiation of an eukaryotic gene requires several components. First, three types of eukaryotic polymerases exist. RNA polymerase II (pol II) is responsible for transcribing protein-coding genes, such as the IFP multi-gene family, and consists of 10 plus or minus

2 subunits. Pol II must be properly aligned at the promoter to correctly initiate transcription. The complex which forms to assure this alignment contains many other factors which are currently under study.

The first step in forming the committed complex is the binding of transcription factor II D (TFIID) to the TATA box in the promoter. TFIID is a complex of proteins itself. It consists of the TATA-binding protein (TBP) and a host of TBP-associated factors (TAFs). The TBP is sufficient to bind to the TATA box and initiate binding of other TFs, but the TAFs confer the transcription regulation by other factors.

After TFIID binds, TFIIB binds and in turn binds TFIIF and pol II. Since pol II does not have the inherent ability to bind to the promoter itself, TFIIF is associated with pol II to facilitate binding. Although the complex now formed is stable, TFIIE,TFIIH, and TFIIJ also bind to the complex. TFIIE is needed to recruit TFIIH, which in turn phosphorylates the carboxy-terminal domain of pol II. It may be that this event signals promoter clearance and turns the initiation complex into an elongation complex, although there is still some controversy surrounding this ATP energy

requirement (Buratowski, 1994; Drapkin et al., 1994). What has been observed is that phosphorylated pol II will not associate with TBP although the non-phosphorylated pol II will (Usheva et al., 1992).

Another factor whose purpose is not completely understood yet is that of TFIIA. This factor appears to bind early in formation of the complex to TFIID. The function of TFIIA has been postulated to be the inhibition of the action of various repressors on the forming transcription complex. By directly binding with the TBP, TFIIA may block the effects of these repressors and allow the other required TFs to associate. Again, this is still under investigation.

Eukaryotic Transcription Regulation

Several *cis*-acting elements have been characterized that influence transcription of eukaryotic genes. These include the promoter elements, enhancers, repressors, and anti-repressors.

Promoters

Promoters are responsible for the basal level of

transcription of a gene. For eukaryotic genes, they must include a sequence to which pol II can associate and thereby allow transcription to ensue. This is typically referred to as the TATA box. Located about 30 base pairs upstream of +1, the transcription start site, the TATA box is recognized by the TBP (see above). It is here that the transcription machinery assembles and is properly aligned and the DNA is unwound.

Promoters have also been described that contain no TATA box or show poor homology to its consensus sequence. In these cases, other *cis*-elements assume the role of the TATA box. Initiator sequences (INRs) overlap the transcriptional start site. INRs do not necessarily show homology across genes and therefore function through various trans-acting factors. The proposed model incorporates an INR-binding protein which serves the initial role of TBP in DNA binding (Hernandez, 1993). The factors that normally bind the TATA box then enter the complex. TBP has been observed to still bind, allbeit weakly, to the -30 region in the absence of the TATA box (Wiley et al., 1992); therefore, positioning would be correct but the efficiency of TBP binding would be

severely compromised.

The promoter region may also contain a CAAT box, which binds a CAAT box transcription factor (CTF), and one or more GC boxes, which bind a protein known as Sp1. The CAAT box is located between -60 and -80 base pairs upstream of the transcription start site +1, and, although it is not palindromic, it may occur in either orientation. In the case of the GC/Sp1 complex, interaction has been observed with TBP. TAF-110 has been shown to bind to Sp1, thus bridging it to the TBP (Hoey et al., 1993; Hernandez, 1993). Many of the TATA-less promoters do contain one or multiple GC boxes, which greatly enhance transcription. Together these promoter elements determine the RNA polymerase specificity and the basal level of transcription.

Enhancers

Enhancers are defined by their ability to promote transcription in association with a promoter and function in a position- and orientation-independent manner. They have been identified thousands of base pairs away from the transcription start site, within introns of genes, and at

the 3'-ends of genes. The question of exactly how enhancers act remains unanswered. One possibility is that the enhancer sequence is recognized by a transcription factor that in turn may stimulate pol II to bind to the promoter region. The other thought is that the enhancer plus its transcription factor may interfere with the histone packaging of the DNA or cause a conformational change in the DNA such that it is now exposed and pol II is able to bind. In actuality, enhancers probably act in some combination of these proposed mechanisms (Voet and Voet, 1990).

Repressors

Like enhancers, repressors function in association with a promoter and in a position- and orientation-independent manner. Because repressors, also known as silencers, act at the level of the promoter, there are many points at which the effect may occur. Silencers may block binding of one of the transcription factors or pol II, or they may inhibit the actual start of transcription. On a more global scale of repression, compaction of DNA inhibits transcription greatly. Relatively little is known about this mode of

regulation in terms of clearcut examples at the various steps, but progress is being made to understand where exactly the control is exerted (Herschbach and Johnson, 1993).

Antirepressors

Antirepressors are a novel mode of regulation (Croston et al., 1991; Stover and Zehner, 1992). Like their counterparts, antirepressors appear to function in a position- and orientation-independent fashion. Genes containing these antirepressor, or antisilencer, sequences have taken regulation to another level. Their purpose is to override the silencing effects of the silencer elements. As a result, transcription rates have been observed to increase. Again, though, antisilencers by themselves will not enhance transcription: silencer elements must be present for the effect to be observed.

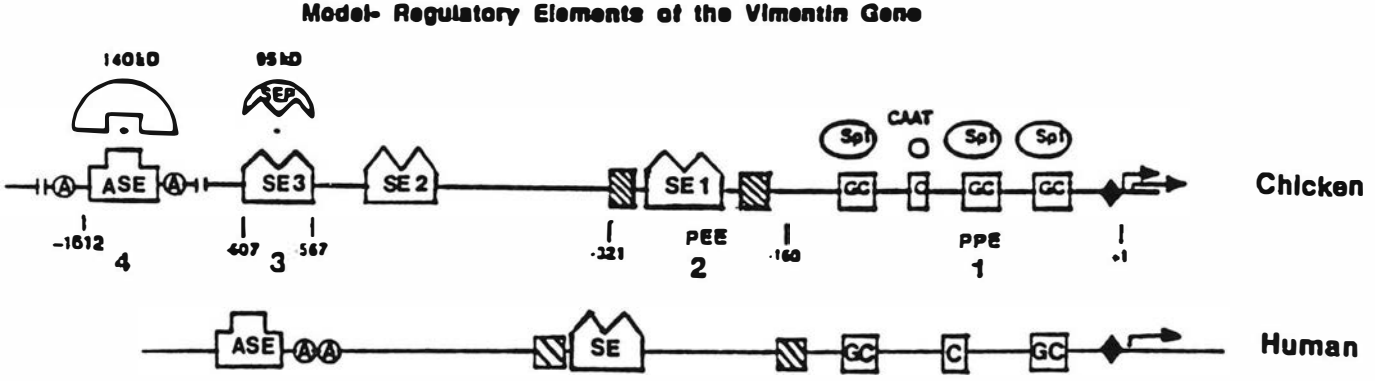
Regulation of Chicken Vimentin

The chicken vimentin gene has been previously isolated (Zehner and Paterson, 1983) and sequenced (Zehner et al.,

1987). To date, regulation of transcription of the chicken vimentin gene has been found to include all four regions as depicted in Figure 1. Comparable elements in the human gene are also shown. The gene is present as a single copy in the chicken genome but yields two classes of mRNAs due to the utilization of different polyadenylation sites in the 3'-end. Covering 8.5 kb of DNA, the gene contains 9 exons. When compared with the hamster vimentin gene, both exon size and position are conserved (Zehner et al., 1987). Sequences required for the regulation of vimentin gene expression are found localized to the 5'-end (Sax et al., 1988; Sax et al., 1989).

The vimentin promoter contains a relatively poor sequence homology to the consensus TATA box, yet this variant is functional in the globin gene. Within the promotor region, however, lie five GC boxes and a CAAT box. The CAAT box is unique in that it lies on the noncoding strand in the reverse orientation with respect to the gene. The GC boxes, which are known to increase transcription rates through binding the transcription factor Sp1, flank the CAAT box on either side. At least three of the five GC

Figure 1: Model of the regulatory elements of the chicken and human vimentin gene. This model is based on the isolation and characterization of various *cis*-acting elements found within the 5'-end of the vimentin gene. The human antisilencer element (ASE) has not yet been isolated but shows sequence homology and has been characterized with its AP1 sites.


Model- Regulatory Elements of the Vimentin Gene
SEP
CAAT
Sp1
Sp1
Sp1
ASE
SE3
SE2
SE1
GC
C
GC
GC
Chicken
-1612
4
407
3
567
-321
PEE
2
-160
PPE
1
+1
ASE
SE
GC
C
GC
Human

sequences have been shown to actively interact with Sp1 (Sax et al., 1988). As a whole, this region, termed the proximal promoter element (PPE), is thought to be responsible for the basal levels of vimentin expression.

Moving along the 5'-end in a linear fashion, the next region contains a silencer element (SE#1) flanked by an enhancer region on either side. The first enhancer element, referred to as proximal enhancer element 1 (PEE1), is 19 bp long and was shown to increase activity about four fold. The other element, termed PEE2 and located downstream of SE#1, is 19 bp in length and only increases activity about two fold. Both PEEs were observed to bind a protein similar in weight and binding specificity to the protein which binds the three SEs (Perkins et al., unpublished data).

The first silencer element identified and characterized was SE#3, approximately 567 bp upstream from the transcription start site. This 40 bp long segment of DNA was observed to bind an approximately 90 kDa protein, which is currently being isolated. The silencer element protein (SEP) was demonstrated not to be Sp1, although the two proteins are similar in size (Farrell et al., 1990). In addition to

the 90 kDa SEP, a 54 kDa protein was observed to associate with the SE and SEP. The 54 kDa protein is currently believed to aid in the binding of the SEP to the SE, but this has not yet been confirmed. By sequence homology, SE#1 and SE#2 were later identified and synthesized. Both were shown to also bind the same 90 kDa protein as SE#3. In addition, when the relative abilities to repress transcription were measured, SE#1 was found to be the strongest. It effected a 75% reduction while SE#2 and SE#3 were each observed to cause approximately a 50% reduction. When all three SEs were taken in conjunction, a 98% reduction over the PPE was observed (Garzon and Zehner, 1994). Additional SE(s) have been postulated to exist in the region -607 to -1612, but nothing has been isolated yet.

The last element characterized thus far is an antisilencer element (ASE) whose purpose is to override the negative effects of the SEs. This 75 bp fragment was shown to bind an approximately 120 kDa protein that is currently being isolated and sequenced. It was demonstrated to restore transcription levels to 100% when associated with the SEs (Stover and Zehner, 1992). Moreover, the FGF induction of

the vimentin gene appears to work specifically through this element and its factors(Carey and Zehner, unpublished data). A comparison of the DNA sequence of the various chicken and human SE, PEE, and ASE elements is shown in Figure 2.

When tandem AP-1 sites were recognized as enhancers in the human vimentin gene (Rittling et al., 1989), a search was made to look for homologous sequences in the chicken vimentin gene. AP-1 is a jun-fos binding site which acts as an enhancer. Although two AP-1 sites have also been identified in the ASE region, the first one has been shown not to confer any antisilencing effects over the three SEs (Stover and Zehner, 1992). Another unique negative element has been discovered upstream of the ASE, in the region between -1612 and -1635. The second AP-1 site, located upstream of this element, has been shown to override the effects of the nearby negative element. This AP-1 site was shown to be serum inducible as well as TGF-β and phorbol ester TPA inducible (Carey and Zehner, unpublished data).

In summary, the regulation of vimentin expression is a complex combination of interactions between several *cis*-elements and *trans*-acting factors. Much work is being done

Figure 2: Comparison of chicken and human sequences. The DNA sequences for the silencer elements (SE), the proximal enhancer elements (PEE) and the antisilencer elements (ASE) show strong homology. Both the chicken and human SE and ASE have been shown to bind the same SEP and ASEP, respectively.

COMPARISON OF CHICKEN AND HUMAN SEQUENCES

Chicken SE#1	**AGGAGCG** $(X)^5$	**GGAGCA**
Chicken SE#3	**AGGAGCG** $(X)^9$	**GaAGCA**
Human SE	g**GG**tc**CG** $(X)^4$	**G**c**A**c**CA**
Chicken PEE1	**AGG**g**GCG** $(X)^3$	**GG**c**GC**c
Chicken PEE2	**AGG**ga**CG**	
Human PEE1	**AGGA**ag**G** $(X)^2$	c**GAG**gc
Human PEE2	**AGG**g**GCG** $(X)^2$	**GGAG**g**A**

(rev) Chicken ASE $^{-1596}$**GCTCTTGGCCACC**$^{-1608}$

Human ASE $_{-881}$**GCTCTTG**t**CC**c**CC**$_{-869}$

to elucidate the various controls. Because the vimentin gene structure is highly conserved across species (Figure 1), it is possible to study the regulation of the chicken gene in human cells such as breast cancer cell lines as well. As will be discussed next, two established cancer cell lines were isolated that differed in their expression pattern of vimentin.

Breast Cancer

According to the American Cancer Society's Cancer Facts & Figures 1993, breast cancer is the second major cause of cancer deaths in women. For noninvasive tumors, the five-year survival rate nears 100% while in cases of distant metastases, the survival rate is only about 18%. Breast cancer is a general term for a whole host of complex tumors affecting the mammary gland epithelium. Some tumors are aggressive and metastatic while others are not nearly as invasive. MDA-MB-231, henceforth referred to as MDA, represents one of the cancers with the poorest prognosis due to its invasive nature. MCF-7 on the other hand has a relatively good prognosis as it is nonmetastatic. Clues to

differences in their behaviors lie in the expression pattern of various proteins.

Though it is still not clear exactly which mutation(s) is responsible for triggering tumor progression, evidence points towards an accumulation of mutations. Some researchers speculate that it may take as many as four or five before the phenotype presents (Fearon and Vogelstein, 1990). At the same time, though, a single mutation may be enough to alter growth patterns and lead to mild manifestations of a diseased state (Edwards, 1993). Whatever the cause, the effect is to alter the machinery governing the growth and differentiation of the cell.

Vimentin Expression in Breast Cancer

As an IFP, vimentin shows a unique expression pattern. Vimentin is normally expressed in cells of mesenchymal origin, therefore being first synthesized in the very early steps of embryogenesis. It is the IFP type found in many established cell lines in tissue culture, is cell cycle regulated, and is induced by serum phorbol esters and

several growth factors, ie. PDGF, TGF-β, and FGF. Because of this expression pattern, it has been suggested that vimentin is the preferred type of IFP found in the freely dividing, highly proliferative cell. Moreover, vimentin expression has been observed in certain poorly-differentiated breast carcinomas (Raymond and Leong, 1989). It has been suggested that these carcinomas may represent cells which have de-differentiated and have reverted back to their embryonic forms because they lose cell-to-cell contact inhibition which results in metastasis (Ramaekers et al., 1983; Gould, 1986). Therefore, vimentin could be used as a prognostic marker for metastatic breast carcinomas (Raymond and Leong, 1989).

Vimentin has clearly been demonstrated to have an altered expression pattern in the metastatic MDA versus the nonmetastatic MCF-7 cell(Sommers et al., 1989; Stover et al., 1994). By Northern analysis, no vimentin mRNA is detectable in the MCF-7 cell line while it is abundant in the MDA line (Figure 3A). This agrees with the aforementioned observation that vimentin expression correlates with metastasis.

In addition, to examine differences in vimentin regulation between the two cancer cell lines, a Southwestern showing the binding activity of both the SEP and ASEP was analyzed (Figure 3B). In the case of the SE#3, the MCF-7 cells showed maximal binding activity while the MDA cells showed no binding of the SEP. The opposite pattern was seen with the ASEP: the MDA line showed maximal binding while the MCF-7 line exhibited only about 60% maximal binding activity. Both binding patterns confirm the vimentin RNA expression pattern observed in the Northern blot.

To explore the possible correlation between metastasis and vimentin expression, it was necessary to identify two breast cancer cell lines which differed in their vimentin expression pattern. Transformed culture cells are typically marked by three traits: a decrease in growth factor requirements, a loss of anchorage dependence, and a loss of contact inhibition. It has been found that most eukaryotic cells introduced into culture will synthesize vimentin, regardless of their tissue origin (Pieper et al., 1992). This is probably due to a combination of the effects of serum and/or growth factors present in the media and the

Figure 3: Northern and Southwestern blot analyses of two different breast cancer cell lines, MCF-7 and MDA-MB-231. *A*, total RNA (3 μg) was analyzed and hybridized with a ^{32}P-labeled human vimentin cDNA as described in Stover et al., 1994. *B*, crude nuclear extracts (50 μg) from both breast cancer cell lines were hybridized with ^{32}P-labeled SE and ASE of equal radiospecific activity and quantitated using scanning densitometry as described in Stover et al., 1994.

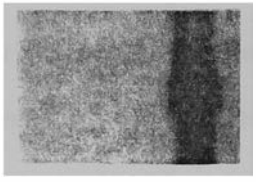
A.

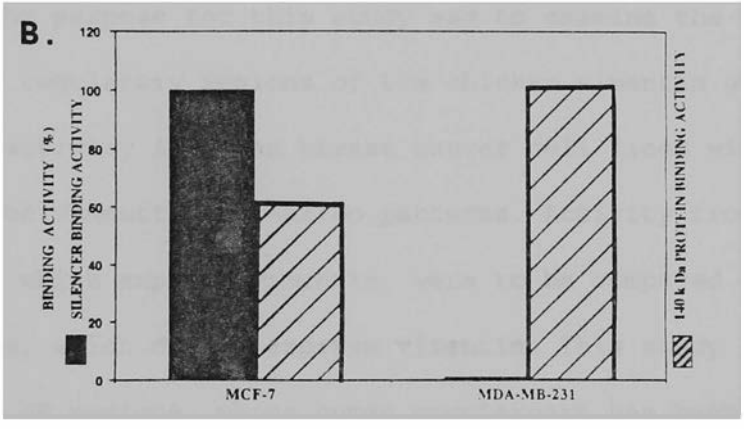
Northern

MCF-7 MDA-MB-231

Hormone Responsive

+ −





tendency of such cells to revert and re-enter the cell cycle. Therefore, it was imperative to find two established breast cancer cell lines which maintained their vimentin expression pattern in culture similar to that of the primary tumor from which they were derived. Presumably these cells were derived from tumors at different stages in progression to the metastatic state. Since expression exists in one line but not the other, it was concluded that these would be appropriate for study of the differential regulation of the vimentin gene.

Rationale

The purpose for this study was to examine the various 5'-end regulatory regions of the chicken vimentin gene for their activity in human breast cancer cell lines with opposite vimentin expression patterns. Activity from MDA cells, which express vimentin, were to be compared with MCF-7 cells, which do not express vimentin. This study focused on the SE regions, whose human counterpart has been isolated and has been shown to bind the same SEP. Also, the human equivalent for the ASE has been found by sequence

comparisons and shows a match of 11 out of 13 bp. Because the chicken and human SEs and ASEs show such homology and bind the same proteins, transfecting the chicken vimentin regulatory regions into the breast cancer cell lines was deemed applicable. In addition, a transfection method that would effectively introduce the DNA constructs into the cell lines was also determined.

MATERIALS AND METHODS

Plasmid Preparation

Plasmid constructs were previously synthesized as indicated (Sax et al., 1988; Farrell et al., 1990; Stover and Zehner, 1992; Garzon and Zehner, 1994). The 5'-end content of plasmid preparations was verified using the appropriate restriction enzymes and visualized on an 8% polyacrylamide gel.

Sequencing

The plasmid construct containing SE#1 was verified by sequencing. The sample was run on an 8% polyacrylamide-7M urea sequencing gel, dried, and visualized on film with an enhancer screen.

Cell Culture, DNA Transfections, and CAT Assays

MDA-MB-231 cells (Thompson et al., 1992) were obtained indirectly from the ATCC (Rockville, MD) through Dr. Eric Westin's lab at the Medical College of Virginia (MDA-1). The cells were maintained in Dulbecco's modified Eagle's medium

(DMEM) supplemented with 10% fetal calf serum (FCS) and 0.1% ampicillin. MDA-MB-231 (MDA-2) and MCF-7 cells were also obtained indirectly from ATCC through Dr. Mary Hendrix's lab at the Pediatric Research Institute, St. Louis University, St. Louis, MO, and were maintained in RPMI 1640 medium supplemented with 10% FCS and 0.1% gentamicin.

All cell types were transfected using the calcium phosphate coprecipitation technique. For each set of transfections, p8CAT was used as a control and pcV-160 was included to measure the basal level of transcription determined by the promoter. Twenty-four hours prior to transfection, cells were plated at a density of 8 x 10^5 cells per 100 mm tissue culture dish in the respective medium. 15 μg of plasmid DNA were introduced to each dish. The media was removed from the dishes, and the cells were washed with 4 ml of fresh media. The DNA-calcium phosphate precipitate was then added dropwise over the cells. The plates were allowed to sit at 25° C for 20 min with gentle shaking every 5 min. Four ml of media was gently added, and the cells were incubated overnight at 37° C. After 24 h, the media was discarded, and the cells were gently washed with 4 ml of fresh media. Three

ml of a 15% glycerol solution were introduced, and the plates were incubated at 37° C for 4 min. The glycerol was then discarded, and the cells were again washed with 4 ml of fresh media. Fresh media (10 ml) was added, and the cells were incubated overnight at 37° C. After 24 h, cells were washed and harvested in phosphate buffered saline, pelleted, and resuspended in 100 μl of 0.25 M Tris. Protein was extracted from the cells by a freeze-thaw cycle repeated three times. The tubes were then centrifuged at 15,000 rpm for 8 min at 4° C. The supernatants were collected, and protein concentrations were determined by the Lowry method (Lowry et al.,1951). The remaining protein was stored overnight at -20° C.

The chloramphenicol acetyltransferase (CAT) assay was performed as described by Gorman et al. (1982). Results were quantified by excising the radioactive spots from the silica plates and using a liquid scintillation counter to determine the ^{14}C content. The dpms obtained from the counter were then converted to pm of acetylated CAT. From this, activity was calculated as the percent acetylated chloramphenicol from the various constructs versus acetylated chloramphenicol from pcV-160. Promoter-driven activity was corrected for using

background levels of the p8CAT vector promoterless construct. The values reported are the average of at least six separate transfections and error bars were calculated using the standard error of the mean (SEM).

Nude Mouse Assay and Invasive Assay

MDA-MB-231 cells obtained from Dr. Eric Westin were checked by injection into nude mice to determine metastatic potential. 2 x 10^6 cells were injected into the mammary fat pad. The mice were sacrificed approximately four weeks later, and the internal organs were retrieved for histological evaluation.

MDA-MB-231 cells obtained from Dr. Mary Hendrix were tested for metastasis by a modified Boyden chamber chemoinvasive assay (personal communication).

RESULTS

DNA Transfections

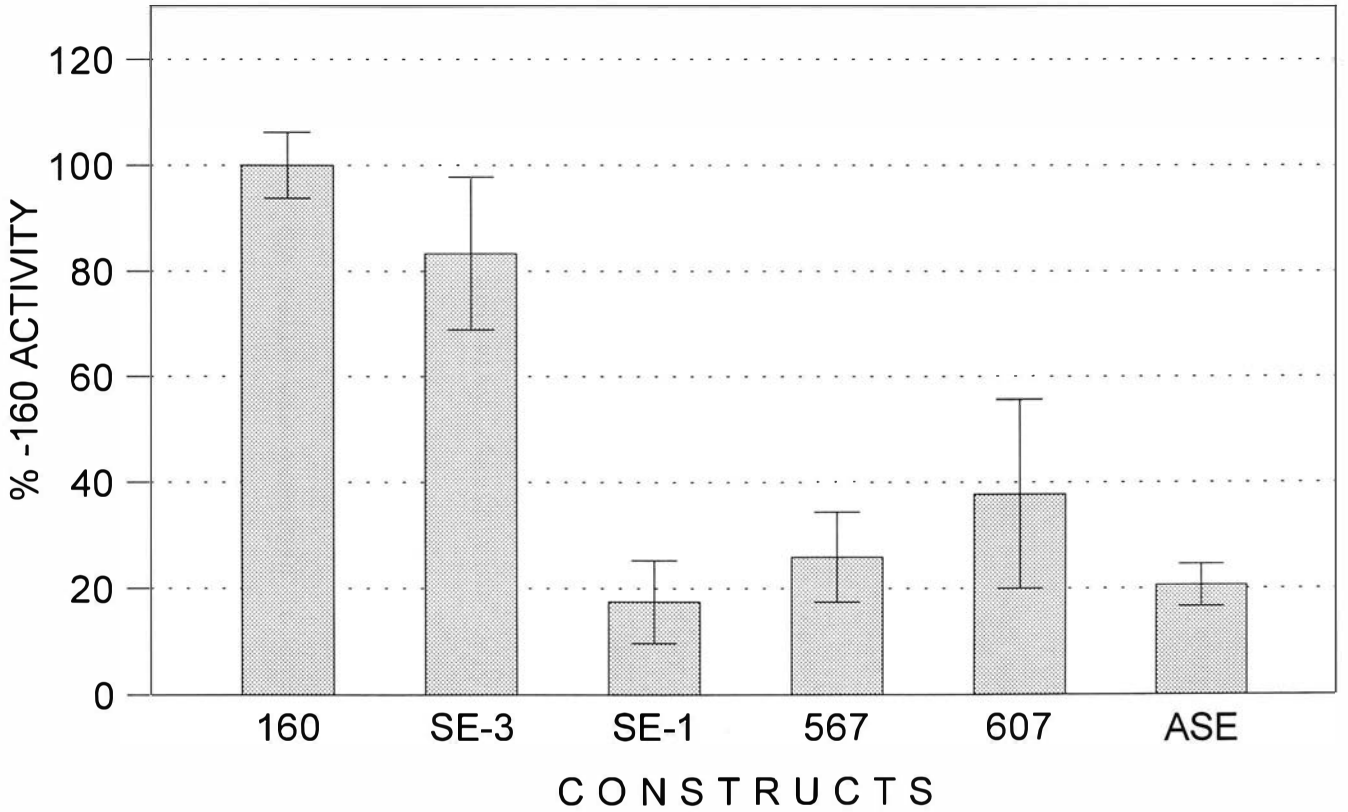
The calcium phosphate coprecipitation technique was determined to be an effective mode of transfection in both the MDA and MCF-7 cell lines and was subsequently used for all trials. It was noted that the MDA-1 cells from Dr. Eric Westin (MCV/VCU) were grown in DMEM while the MDA-2 and MCF-7 cells from Dr. Mary Hendrix (St. Louis University) were grown in RPMI-1640. Upon introducing the $CaPO_4$ solution to the cells grown in RPMI-1640, it was observed that a white precipitate formed. Also, it was observed that many of the cells had subsequently lysed.

MDA Data

The first cells examined were those obtained from Dr. Westin's lab (MCV/VCU). As can be seen in Figure 4, transcriptional activity in all cases is reported as the percent pcV-160 activity, which is the basal promoter

Figure 4: Transient transfections of various chicken vimentin 5'-end CAT constructs in MDA-MB-231 cells (1). Transfection was accomplished using the calcium phosphate coprecipitation technique as described in Materials and Methods. Values reported are relative to the basal promoter pcV-160 activity, and error bars were calculated using the SEM.

PERCENT -160 ACTIVITY
MDA 1

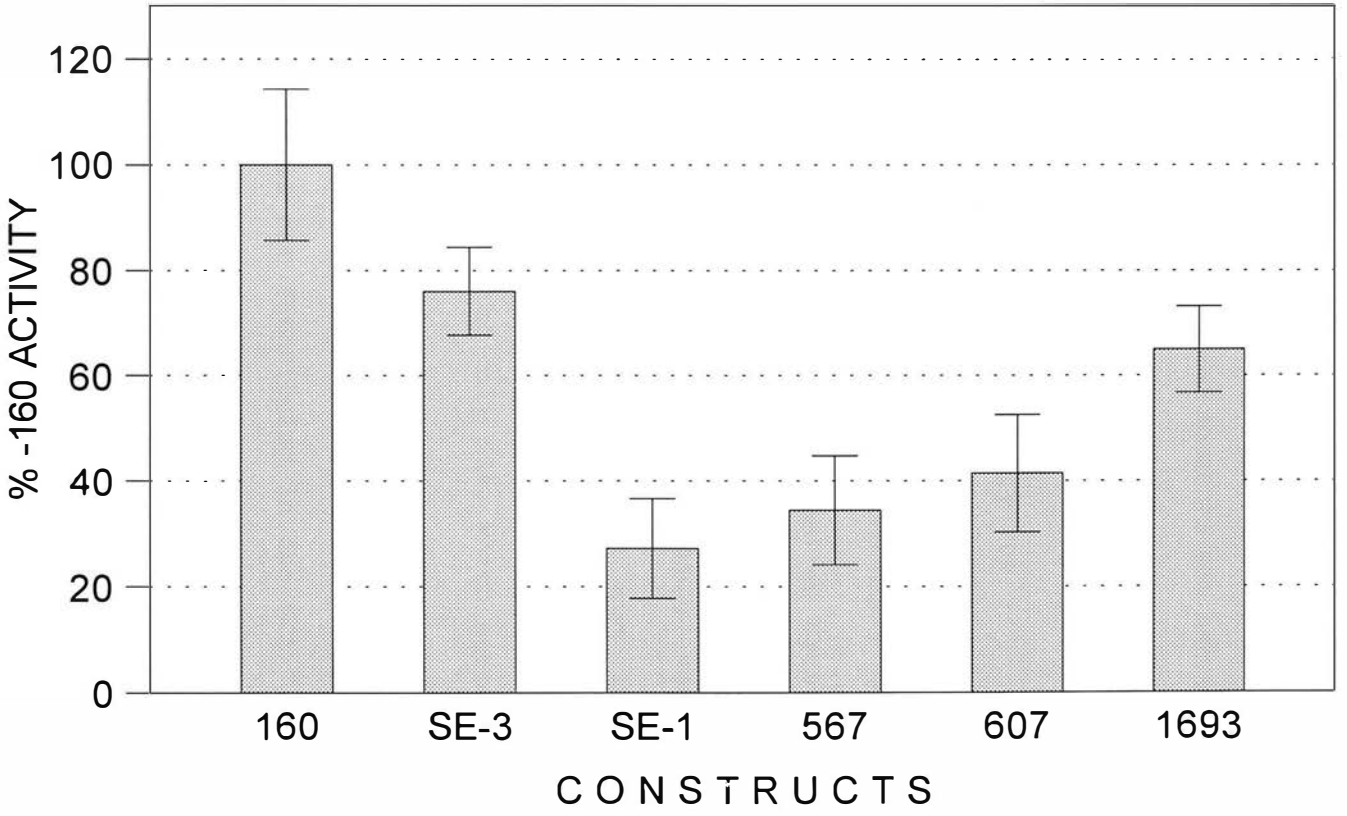


activity. Construct pcV-567 showed only about 25% activity relative to the basal promoter. Since this element contains the first two SEs, the 75% repression seen was not surprising. What was surprising was the activity of pcV-607, whose sequence merely includes that of SE#3. Rather than reducing activity further, inclusion of the third SE showed about the same activity or maybe slightly more activity than pcV-567. Remembering that these MDA cells do express vimentin, it may be here that part of the regulation of vimentin expression is affected. Taken individually, both SE#1 and SE#3 fused directly to pcV-160 showed quite different activity levels. SE#1 appeared to repress activity by about 75% while SE#3 repressed very little, if at all. When the ASE was examined, it also showed very little activity. Exhibiting only 25% of the total activity, the ASE does not appear to be the only factor responsible for vimentin expression in these cells.

The second line of MDA cells was examined in comparison to both the previous MDA cells and the MCF-7 cells. Again, in Figure 5, the data reported is relative to the basal promoter activity. Constructs pcV-567 and pcV-607 showed little difference in their activity. This is in agreement with the

Figure 5: Transient transfections of various chicken vimentin 5'-end CAT constructs in MDA-MB-231 cells (2). Transfection was accomplished using the calcium phosphate coprecipitation technique as described in Materials and Methods. Values reported are relative to the basal promoter pcV-160 activity, and error bars were calculated using the SEM.

PERCENT -160 ACTIVITY
MDA 2



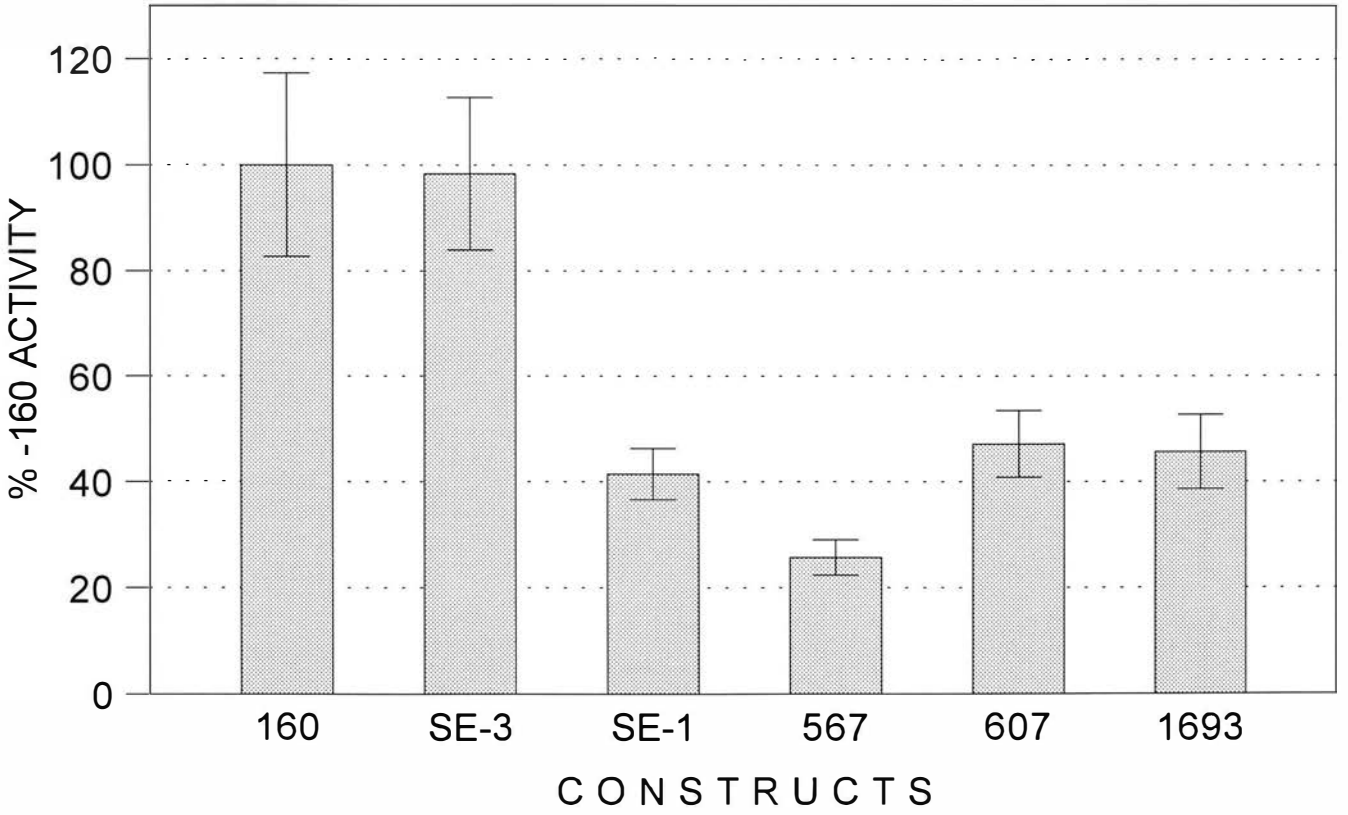
observations from the first set of cells. The same is true for SE#1 and SE#3 fused directly to the promoter. SE#1 shows only about 25% activity, while SE#3 shows about 75-80% activity. On the other hand, construct pcV-1693, including the ASE and distal AP1 site, appeared to be functioning to a greater extent, yielding activity of approximately 70%.

MCF-7 Data

The results of DNA transfections into the MCF-7 cells are shown in Figure 6. Construct pcV-567 showed an activity of about 25%, similar to that observed for both sets of MDA. On the other hand, pcV-607 showed activity of about 50%. This appears to demonstrate that inclusion of SE#3 actually increases activity rather than repressing it. SE#1 fused to the promoter showed activity of only 50%, which agrees with earlier studies (Garzon and Zehner, 1994). SE#3 fused to the promoter essentially yielded full activity of 100%. It would appear that this SE is not functioning in the MCF-7 cells, which does not agree with the fact that these cells do not express vimentin. The ASE showed only about 50% activity, which theoretically may not be sufficient to overcome the silencing effects.

Figure 6: Transient transfection of various chicken vimentin 5'-end CAT constructs in MCF-7 cells. Transfection was accomplished using the calcium phosphate coprecipitation technique as described in Materials and Methods. Values reported are relative to the basal promoter pcV-160 activity, and error bars were calculated using the SEM.

PERCENT -160 ACTIVITY
MCF 7



Nude Mouse Assay

All four of the mice injected with the MDA cells from Dr. Eric Westin developed macroscopic metastases. Tumors were directly observed in the liver, spleen, mesenteric lymph nodes, lungs, kidneys, intestines, and the ovaries.

DISCUSSION

The purpose of this study was two-fold. The first objective was to determine a transfection protocol that would effectively introduce chimeric DNA into the two cancer cell lines. The second objective was to study possible regulatory differences between the two cancer cell lines by comparing the resultant CAT activity from various 5'-end constructs. The MDA and MCF-7 cell lines exhibit opposite expression patterns of vimentin mRNA and were therefore chosen to try to determine where in the regulation of vimentin gene transcription the difference in expression arises. As will be discussed, several possibilities exist to explain the observed results.

The first step in the study was to determine a transfection protocol that would effectively introduce the DNA into both cell lines. Past experience in this laboratory with DNA transfections has shown the $CaPO_4$ coprecipitation technique to be the most effective. This technique was

therefore attempted first, and the test trial showed CAT activity, which meant that the DNA was indeed being taken up and expressed by the cells. Throughout the study, the $CaPO_4$ technique was then used to introduce the various gene constructs into the cells.

One noted observation was the appearance of a white precipitate that formed upon mixing of the $CaPO_4$-DNA solution and the RPMI media. Twenty-four hours later, many of the cells appeared to have lysed. CAT activity was still obtained, so the cells that survived were still able to take up and express the transfected DNA. The stocks of DNA constructs were determined not to be the cause, because the precipitate formed only when the $CaPO_4$ solution and the RPMI media were mixed in a dish. DMEM did not show any adverse reactions like the RPMI. The only apparent difference in RPMI and DMEM is that RPMI contains inositol. The DMEM actually contains more $CaCO_3$, which we originally thought might be the source of the precipitate. For future studies, the cell lines can be transfected by the $CaPO_4$ coprecipitation technique, but they will need to first be conditioned to another medium, preferentially DMEM since that did not show any evidence of

side reactions.

Part two of this study was to examine the expression patterns observed upon introduction of the various regulatory regions fused to the CAT reporter gene into the metastatic and nonmetastatic cell lines. Stover et al. determined that MDA cells do express vimentin while MCF-7 cells do not. From a Southwestern blot, Stover et al. showed 100% binding activity of the SEP to SE#3 in MCF-7 cells while almost no SEP bound to SE#3 in MDA cells. When they examined the ASE, they found 100% binding of the ASEP in MDA cells while only about 60% binding of the ASEP was observed in MCF-7 cells. The differences in these binding activities supported the observed expression patterns of vimentin in these two cell lines; therefore, constructs containing these regulatory elements were used for the study.

p8CAT, a derivative of pEMBL8 (Dente et al., 1983), contains the bacterial CAT gene and an ampicillin resistance gene. Expression of vimentin was therefore inferred from the measured CAT expression. In all cases, the activity of the constructs was reported relative to the pcV-160 construct, which contains the basal promotor element of the vimentin

gene.

MDA cells obtained from Dr. Eric Westin (MCV/VCU) were tested first, but since the MDA cells obtained from Dr. Mary Hendrix exhibited a similar activity profile, they will be discussed simultaneously. SE#3, which was used in the binding studies of the SEP, showed little, if any, repression. This agrees with the previous data that the SEP does not bind in the MDA cells and therefore vimentin is expressed. SE#1, on the other hand, showed 75-80% repression of activity and appeared to be functioning. In support of these observations, pcV-567 and pcV-607 showed similar patterns. Construct pcV-567 contains SE#1, SE#2, and other negative regulatory elements which have not yet been fully identified or characterized. This particular construct also showed about a 75% repression in activity. By adding on SE#3 in pcV-607, though, no additional repression activity was observed. This meant that SE#3 was not functional in MDA cells. The discrepancy was in SE#1 activity: it appeared to be functional, although we know that MDA cells do not express vimentin. One explanation for this is that the SEP is mutated in such a way so that it may still be able to bind to SE#1 but no longer to SE#3. From

Figure 3, one can see that the SE sequences are not identical, which may affect binding ability. SE#1 may be the preferred sequence and thus bind the protein more tightly. When the protein is mutated, the binding to SE#3, which is presumably weaker to begin with, may be abolished and thereby inactivate SE#3. The other possibility is that the 54 kDa protein, thought to be required for functional silencer protein binding, may be mutated and affecting SEP binding. Since very little is known about the role of the 54 kDa protein at this time, it is difficult to assess the importance of this protein to SEP function. On the other hand, a combination of mutations in both proteins may contribute to aberrant vimentin expression.

In studying the ASE, activity was only restored about 25% in the first set of MDA cells. Previous studies in mouse L-cells have shown that the ASE can restore about 80% of the activity, but the activity we observed in the MDA cells was substantially lower. As was previously determined, the ASE only functions in the presence of the SEs. What is not clear is exactly how this ASE works. If the SEP must be bound to the three SEs in order to initiate binding of the ASEP to the ASE,

then having only one SEP bound to SE#1 may not be enough to fully activate the ASE in overriding SE#1. In other words, the conformation of the DNA and/or protein-protein interactions may play a role in how the ASE acts. Further studies are needed to determine how the ASE acts *in vivo*. However, in analyzing the results from the second set of MDA cells, the ASE appeared to be functioning. The difference between the ASE constructs used in MDA 1 and MDA 2 is about 80 bp. Included in the 80 bp is a second AP-1 site. At least in the case of human vimentin, this second AP-1 site was shown to have a significant antisilencing effect (Rittling et al., 1989). In conclusion, the second AP-1 site may play a crucial role in establishing the full antisilencing effect in the MDA cell line such that vimentin is highly expressed.

The profile observed for the MCF-7 cell line is indicative that vimentin's regulatory elements are still present. Since these cells do not normally express vimentin, though, the gene may be under more global mechanisms of control such as methylation and DNA condensation. The similarity in the MCF-7 versus the MDA profiles suggests that the *trans*-acting factors are still functional. For the native

gene to be turned on, other events must occur before the *trans*-acting factors may interact with the *cis*-acting elements.

In summary, the results observed for SE#3 substantiated the earlier findings of Stover et al. that functional SE#3:protein binding was not occurring in the metastatic MDA cell line. Surprisingly, SE#1 appeared to be functional. As for the ASE, the first ASE construct did not appear to function as expected, but this was probably due to the absence of the distal AP-1 site. The fact that the second AP-1 site in human vimentin has been shown to be critical for antisilencing activity further supports this. When the second chicken AP-1 site was included, the antisilencing effect was restored and again substantiated the earlier findings of Stover et al. The results from the MCF-7 cell line suggest that all regulatory factors are present and functional, but the native gene is probably under more global mechanisms of control which completely repress gene transcription. The key to understanding the differences in regulation between these two cell lines will lie in understanding the SE and the ASE. Further characterization of how the SEP and 54 kDa protein

interact and how the ASEP is able to override the SE effects will provide the clues as to how these regulatory differences affect gene transcription.

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